

# Parathyroid hormone-related peptide can regulate the growth of human lung cancer cells, and may form part of an autocrine TGF- $\alpha$ loop

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Parathyroid hormone-related peptide (PTHrP) and transforming growth factor- $\alpha$  (TGF- $\alpha$ ) were found to stimulate proliferation of human lung cancer cells (BEN-57). TGF- $\alpha$  stimulated PTHrP secretion from these cells. The polyclonal antisera raised against PTHrP significantly inhibited the growth of BEN-57 cells, and also the proliferation induced by TGF- $\alpha$ . Treatment of cells for up to 10 days with either a PTHrP receptor antagonist (PTHrP(7–34)) or PTHrP antiserum significantly inhibited the subsequent growth of these cells. We suggest that PTHrP may be a component of a complex autocrine loop involving TGF- $\alpha$ .

PTHrP; TGF- $\alpha$ ; Lung cancer; Autocrine

## 1. INTRODUCTION

The production of both inhibitory and stimulatory growth factors is a likely mechanism leading to the autocrine regulation of normal cellular proliferation. While these factors are known to bind to specific receptors the exact manner in which they exert their effect is less certain. However, induction of other cellular oncogenes, that are either permissive for cell division or that trigger it, may be one way in which they act. In addition, over-expression of certain growth factors may be part of the initiation process leading to tumour formation [1].

The recently discovered parathyroid hormone-related peptide (PTHrP) has been implicated as the major cause of the syndrome of humoral hypercalcaemia of malignancy [2]. In this condition, exhibited by almost 20% of cancer patients, production of PTHrP by tumours leads (through the molecule's parathyroid hormone-like bioactivity) to a significantly elevated plasma calcium level, so complicating further the clinical management of the tumour. While the production of ectopic hormones by tumours is not uncommon, it has begun to emerge that some of these humoral factors may (in addition to possessing systemic hormonal activity) stimulate tumour growth in an autocrine manner [3].

Expression of PTHrP is not confined to tumour cells, having been isolated in a variety of normal human adult [4] and foetal tissues [5–7]. The role it plays in adult physiology is unclear but in the case of the foetus it has been suggested that PTHrP may be responsible for the

maintenance of the foetus-directed transplacental calcium gradient [8] and, additionally it may play a role as a foetal growth factor [7]. Although PTHrP may possess growth factor-like bioactivity, it is uncertain whether it exhibits bioactivity similar to transforming growth factor- $\beta$  (TGF- $\beta$ ) [9,10]. The peptide does, however, modulate the effect of TGF- $\beta$  on DNA synthesis by bone cells [11]. The secretion of PTHrP by a human osteosarcoma cell line is enhanced by epidermal growth factor [12] as is secretion by human keratinocytes [13]. Furthermore, addition of PTHrP to human keratinocytes has been suggested to inhibit proliferation and induce terminal differentiation [14].

Human foetal lung is a plentiful source of PTHrP [5], as are many cases of carcinoma of the lung [4]. In contrast, normal adult human lung does not appear to express the peptide. We report here the results of experiments designed to investigate whether or not PTHrP can function as an autocrine growth factor for a human lung cancer cell line (BEN-57), *in vitro*.

## 2. MATERIALS AND METHODS

### 2.1. Peptides and antisera

PTHrP(1–34) and -(7–34) were obtained from Peninsula Laboratories (Merseyside, UK). Recombinant human transforming growth factor- $\alpha$  (TGF- $\alpha$ ) was the gift of Dr. R. Derynck (Genentech Inc., CA, USA).

Polyclonal antisera raised in rabbits against PTHrP(1–34) and -(56–86) was the gift of Drs. G.V. Segre and H. Juppner (Massachusetts General Hospital, Boston, USA). The specificity and use of these antisera in immunocytochemistry have been characterised previously [6].

### 2.2. Cell culture

Human lung cancer cells, BEN-57, were obtained from the cell

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bank, Sir William Dunn School of Pathology (University of Oxford, UK). These cells were grown as described previously [15] in DMEM/199 medium (1:1) supplemented with glutamine and 2% foetal calf serum.

Human renal cell carcinoma cells (SKRC-1), were provided by Dr. N.H. Bander (Memorial Sloan-Kettering Cancer Institute, New York, USA), and were grown as described previously [16]. Transformed human keratinocytes (SVK14) were supplied by Dr. T. Kamalati (Division of Biomedical Sciences, Kings College, London, UK) and were grown as described previously [17].

### 2.3. PTHrP assay

A two-site immunoradiometric assay for PTHrP(1-86) was kindly performed by Dr. W. Ratcliff (Queen Elizabeth Hospital, Birmingham, UK) as described previously [18].

Cells were maintained in Nunc 75 cm<sup>2</sup> tissue culture flasks. In experiments, cells were seeded at the required density in Nunc 24-well plates and treated with either peptides or antisera for the desired time period. Cells were then trypsinized and counted using an Elzone Particle Counter, model PC. Statistical significance between control and treated groups was assessed using Student's *t*-test. Data are represented as mean  $\pm$  S.E.M.

## 3. RESULTS

BEN-57, SKRC-1 cells and human keratinocytes, are known to secrete PTHrP. By adding or removing PTHrP from cultures of growing cells the effect of endogenously secreted peptide on cell growth was assessed.

PTHrP(1-34) was found to be mitogenic for BEN-57 cells (Fig. 1), but this effect was produced only at a concentration of 100 nM, and as such considerably in excess of that found in culture medium. Twenty-four hour conditioned media contained PTHrP at a concentration of approximately 65 pM (Fig. 2b). TGF- $\alpha$  was mitogenic to BEN-57 cells and stimulated growth at an optimal concentration of approximately 1 pg/ml (Fig. 2a). To see whether or not TGF- $\alpha$  stimulated PTHrP release from BEN-57 cells, cultures were incubated in

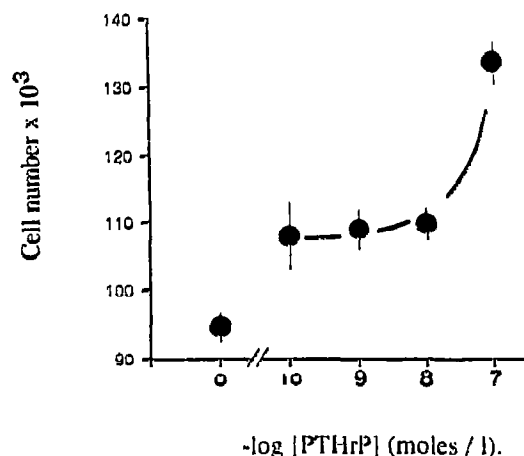


Fig. 1. PTHrP(1-34) is mitogenic to BEN-57 cells but only at high doses. Cells were seeded at a concentration of  $3 \times 10^4$  cells per well. After 12 h, cells were cultured with medium, either alone or containing PTHrP(1-34) at the concentration indicated. The culture media was changed daily, and after 3 days the cells were trypsinized and counted. (Points represent the mean  $\pm$  SEM;  $n=4$ ).

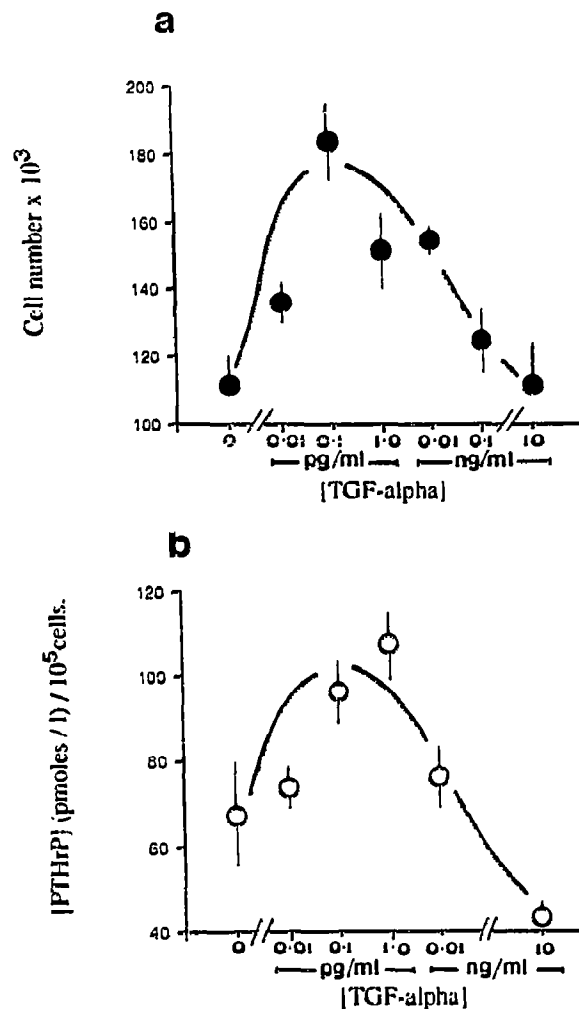


Fig. 2. Effect of TGF- $\alpha$  on (a) growth of BEN-57 cells, and (b) PTHrP secretion by BEN-57 cells. (a) Cells were seeded at a concentration of  $4 \times 10^4$  per well. After 12 h, cells were cultured with medium containing TGF- $\alpha$  at the concentration indicated. Media was changed daily and after 3 days the cells were trypsinized and counted. (Points represent the mean  $\pm$  SEM;  $n=8$ .) (b) Cells were seeded at a concentration of  $10 \times 10^4$  per well and 12 h later the culture media was replaced with 0.5 ml of medium containing TGF- $\alpha$  at the concentration indicated. After a further 24 h medium was removed and assayed for PTHrP (1-86). (Points represent the mean  $\pm$  SEM;  $n=4$ .)

the presence of TGF- $\alpha$  for 24 h and the medium subsequently assayed for immunoreactive PTHrP. PTHrP secretion was stimulated by TGF- $\alpha$  (Fig. 2b) – again at an optimal concentration of approximately 1 pg/ml.

Addition of antisera raised against both PTHrP(1-34) and -(56-86) was found to significantly inhibit cell growth after three days (Fig. 3a shows the mean effect of both antisera). This effect did not appear to be a non-specific or cytotoxic one as preimmune serum was without effect, and addition of the same antisera stimulated the growth of transformed human keratinocytes (Table I). Furthermore, as Fig. 3b shows, it was possible

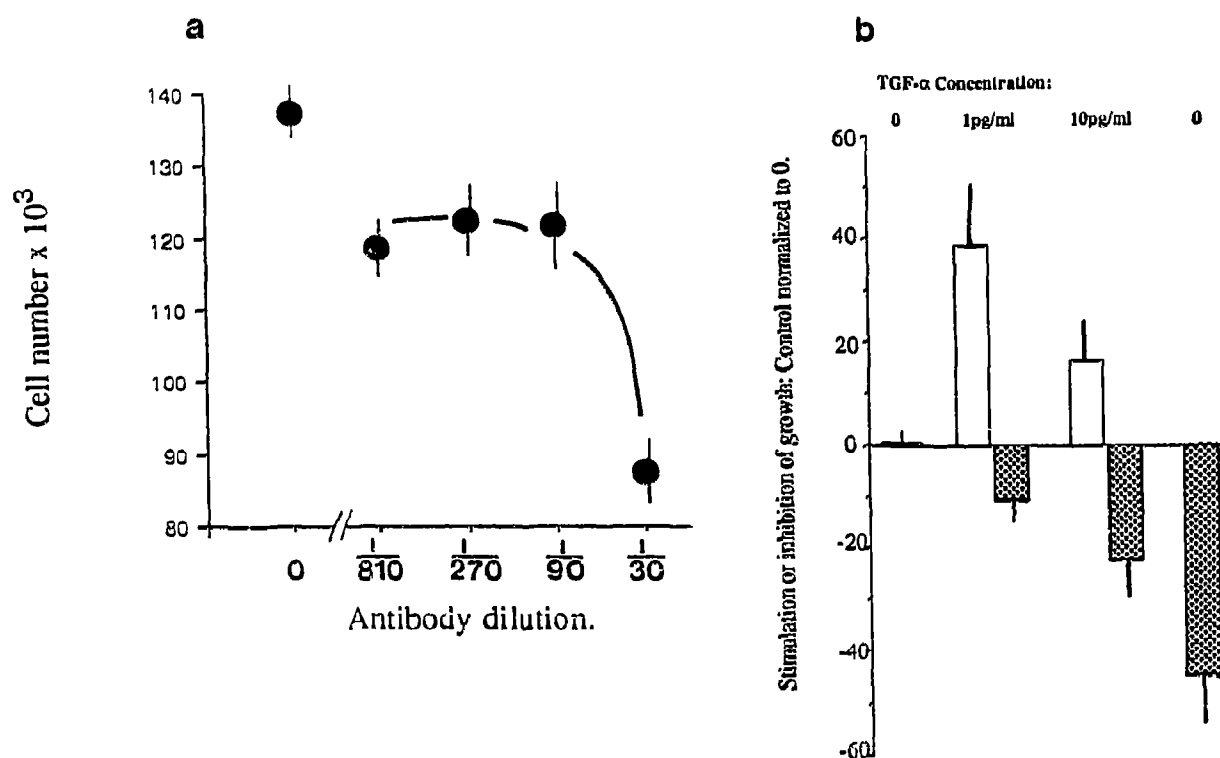


Fig. 3. Polyclonal PTHrP antisera inhibit the growth of BEN-57 cells. (a) Cells were seeded at  $4 \times 10^4$  per well and 12 h later the culture medium was changed for one containing antisera raised against either PTHrP(1-34) or -(56-86). After 48 h cells were trypsinized and counted. (Points represent mean of both antisera  $\pm$  SEM;  $n=8$ .) (b) Cells were seeded at a concentration of  $10 \times 10^4$  per well and 12 h later the culture medium was replaced with 0.5 ml of medium containing TGF- $\alpha$  at the concentration indicated (open bars), or with a medium containing TGF- $\alpha$  and PTHrP(1-34) antisera at a concentration of 1/30 (speckled bars). After 3 days cells were trypsinized and counted. (Points represent the mean % stimulation or inhibition compared to untreated controls  $\pm$  SEM;  $n=3$ .)

to reduce the inhibitory effect of the antisera on BEN-57 cells by inclusion of TGF- $\alpha$  in the media which, as just described, was found to enhance PTHrP secretion and cellular proliferation.

Treatment of cells with the PTHrP receptor antagonist (PTHrP(7-34)) for 3 days did inhibit growth, but not to the same extent as treatment with antisera. 1  $\mu$ M of PTHrP(7-34) inhibited growth by approximately 20%. However, long-term treatment of BEN-57 cells, for up to 10 days, with either PTHrP(56-86) antisera or PTHrP(7-34) markedly inhibited cell growth (Table I). When these cells were trypsinized and seeded into culture plates in medium without either antagonist or antisera their subsequent growth was significantly inhibited (Fig. 4) throughout the following period. There was no obvious change in cell morphology following long-term treatment with either antisera or antagonist.

#### 4. DISCUSSION

The data presented in this study demonstrates, for the first time, that PTHrP can modulate the growth of human lung cancer cells, possibly in an autocrine manner. This is suggested by the fact that PTHrP, albeit at

relatively high concentrations, triggers cell growth, and the antibody to PTHrP inhibits proliferation. TGF- $\alpha$  stimulates cell proliferation and also, over the same dose range, PTHrP secretion. The possibility that TGF- $\alpha$  exerts, in part, its effect on cell proliferation via PTHrP is supported by the finding that the antibody to PTHrP inhibits this TGF- $\alpha$  induced response.

It has been realised for a number of years that withdrawal of growth factors from some tumour cells results in inhibition of growth of these cells and programmed cell death, or apoptosis [19-21]. Recently, it has been demonstrated that removal of interleukin-3 from hemopoietic precursor cells results in cell death as distinct from inhibition of proliferation [22]. The data presented here would suggest that PTHrP may be mitogenic to BEN-57 cells: it is not certain whether PTHrP protects these cells from apoptosis, nor is it known whether it stimulates expression of so-called 'competence genes' such as *c-myc*, which in turn mediate cell division. What is clear, however, is that PTHrP is required for the maintained proliferation of BEN-57 cells in vitro.

The culture medium in which BEN-57 cells proliferated for 24 h contained 65 pM PTHrP. If this level of PTHrP is sufficient to trigger mitosis then it is reasona-

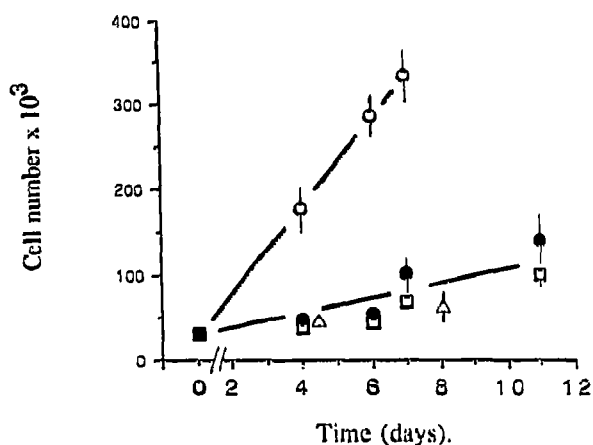


Fig. 4. Treatment of BEN-57 cells with polyclonal PTHrP antisera or the PTHrP antagonist PTHrP(7-34) inhibits subsequent cell growth. Cells were seeded at a concentration of  $2.0 \times 10^5$  in  $3.5 \text{ cm}^2$  Petri dishes. After 12 h, the culture media was replaced with one containing no additives (○), or with one containing antisera raised against PTHrP(56-86) (□) diluted 1/20, or with one containing  $1 \mu\text{M}$  PTHrP(7-34) (●). Media were changed on alternate days over the following 10 days (or in a separate experiment with  $1 \mu\text{M}$  PTHrP(7-34) for only 6 days; △). Cells were then trypsinized and re-seeded at a concentration of  $3 \times 10^4$  per well in 24-well plates in media lacking antisera and antagonist. Cells were counted and the media changed 4, 6, 7 and 11 days later. (Points represent mean  $\pm$  SEM;  $n=3$ .)

ble to question the physiological significance of a concentration of 100 nM PTHrP required to further trigger proliferation. One possibility is that since BEN cells secrete a PTHrP of at least 108 amino acids, the most potent mitogenic activity may reside within the C-terminal portion of the molecule rather than in the N-terminal 34 amino acid fragment used here.

Cell division was inhibited by treatment with either a PTHrP antagonist or PTHrP antisera; this did not appear to be a non-specific effect on the part of the antisera since it appeared to stimulate the growth of transformed human keratinocytes, and the effect could be 'tired out' by inclusion of TGF $\alpha$  in the medium. The fact that PTHrP antisera stimulated the growth of human keratinocytes, which secrete PTHrP, would suggest that the peptide tonically inhibits the growth of these cells. The data shown here that PTHrP can stimulate the growth of some cells and inhibit that of others is similar to the effects of TGF- $\beta$  which can be both inhibitory or stimulatory, depending upon the cell type studied. The observation of reduced proliferation following treatment for 6 or 10 days (Fig. 4) with either of these agents was surprising but was consistent with a previous report of the effect of TGF- $\beta$  on endothelial cells [23], and more recently to the effect of a substance P analogue on lung tumor growth [24]. Everard and colleagues [24] demonstrated that treatment of various small cell lung cancer cell lines with a substance P analogue for as little as 4 h markedly inhibited the subse-

quent growth of these cells. It is possible that tumor cells, if deprived of various critical growth factors, undergo a form of programmed differentiation that may or may not end in apoptosis. Antibodies to bombesin [25], TGF- $\alpha$  [26] and the oncogene product *neu* [27] have been shown to inhibit tumour growth both in vitro and in vivo. Immunotherapy, utilizing antisera raised against various critical growth factors, is of potentially great value in the treatment of cancer and a clinical trial using antibodies to bombesin has been undertaken [28].

We have demonstrated here that 'secretion' of PTHrP by BEN-57 cells is enhanced by treatment with TGF- $\alpha$  which also stimulates cell growth. This observation, and those of others [13,14], would suggest that PTHrP may form part of a complex growth factor 'autocrine loop' such as has been suggested to exist for TGF- $\beta$  and platelet derived growth factor [29]. The control of cellular differentiation and proliferation remains, for the most part, a mystery. While TGF- $\beta$  has been suggested to be the primary differentiation inducing factor in serum for normal human bronchial epithelial cells [30], the picture of cell division seems more complicated as more growth-like factors, such as PTHrP, are discovered. We have demonstrated here that PTHrP, whose role in the aetiology of human lung cancer was considered to be confined to the systemic induction of hypercalcaemia is, in addition, a factor modulating the prolifer-

Table I  
PTHrP antiserum has different effects on proliferation

Cell line	Treatment	
	-	+
<i>Antisera</i>		
SKRC-1	87,000	35,000
SVK14	85,000	140,000
BEN-57 <sup>a</sup>	140,000	87,000
BEN-57 <sup>b</sup>	1,600,000	360,000
<i>Antagonist</i>		
BEN-57 <sup>c</sup>	700,000	270,000
BEN-57 <sup>d</sup>	1,600,000	400,000

SKRC-1 cells were seeded at  $3 \times 10^4$  cells per well and treated for 2 days with antisera raised against PTHrP(1-34). SVK14 cells were seeded at  $4 \times 10^4$  cells per well and treated for 2 days with antisera raised against PTHrP(1-34). BEN-57 cells were treated in four different ways, and these are designated BEN-57<sup>a</sup> through to BEN-57<sup>d</sup>. In each case the culture medium was replaced every 2 days. BEN-57<sup>a</sup>: cells were seeded at a concentration of  $4 \times 10^4$  per well and treated for 2 days with antisera raised against PTHrP(1-34). BEN-57<sup>b</sup>: cells were seeded at a concentration of  $2 \times 10^5$  per Petri dish and treated with antisera raised against PTHrP(56-86) for 10 days. BEN-57<sup>c</sup>: cells were seeded at a concentration of  $2 \times 10^5$  per Petri dish. They were then treated with the PTHrP receptor antagonist PTHrP(7-34) for 6 days. BEN-57<sup>d</sup>: cells were seeded at a concentration of  $2 \times 10^5$  per Petri dish. They were then treated with the PTHrP receptor antagonist PTHrP(7-34) for 10 days. The data shown in the table are the numbers of cells per well from a single experiment, and are representative of 4 experiments.

eration of human lung cancer cells. If antibodies and antagonists to this factor prove to have a similar effect *in vivo* they may have an important role in the therapy of some PTHrP producing tumours.

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